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Surveillance and Elimination of Bacteriophage Contamination in an Industrial Fermentation Process

James A. Zahn and Mathew C. Halter

Abstract

Commercial fermentation processes are often vulnerable to bacteriophage due to the lack of genetic diversity and use of high cell density cultures. Bacteriophage infections in these fermentations can have adverse impacts on operability of the production facility and product quality and prevent recovery of valuable bioproducts in the downstream process. Prevention strategies have been developed and optimized through feedback from bacteriophage diagnostic tests, which inform improvements to process design for elimination of entry points, as well as modification of the biocatalyst to reduce or eliminate bacteriophage virulence. In this chapter, we provide case studies for successful elimination of bacteriophage virulence via host modifications, including bacteriophage binding-site modifications on the outer membrane of an *Escherichia coli* production host, used for commercial manufacture of 1,3-propanediol, as well as application of CRISPR-associated protein 9 (Cas9) for bacteriophage immunity. Finally, we report application of bacteriophage diagnostic methods to fully characterize and eliminate bacteriophage entry points in a commercial fermentation process.

Keywords: bacteriophage, white biotechnology, industrial fermentation, CRISPR, Cas9, phage

1. Introduction

Increasing awareness of our dependence on petroleum, coupled with the negative effects of this dependency on oil supply, price volatility, and gas emissions, is the driver behind the growing global market for biorenewable chemicals made through white biotechnology processes. In 2011, revenue from biorenewable chemicals exceeded \$2.4 billion, and revenue continues to grow at a compound annual growth rate of 14.8%, with glycerin and lactic acid accounting for 79% of the market share [1]. Growth for biorenewable chemicals that are used as monomers the manufacture of bioplastics represents a fast-growing segment of this industry [1].

One example of a biorenewable chemical that has displaced petrochemical manufacturing routes is 1,3-propanediol or BioPDO™. This chemical had historically been manufactured using petroleum-derived ethylene oxide or acrolein [2, 3]. In 2006, DuPont Tate & Lyle Bio Products, a joint venture formed between DuPont and Tate & Lyle, commercialized an aerobic fermentation process for production of 1,3-propanediol (BioPDO™) from glucose derived from yellow dent field corn.

A life cycle analysis (LCA) showed that the BioPDO™ manufacturing process consumed 42% less energy and emitted 56% less greenhouse gas emissions than petrochemical manufacturing routes. BioPDO™ is included as a raw material in polyester manufacture for textiles, carpet (Sorona®), and thermoplastic resins (Sorona® EP). Furthermore, BioPDO™ has direct uses in foods, cosmetics, and personal care applications through the Zemea® brand, and heat transfer, homopolymer, polyurethane, and other industrial applications through the Susterra® brand [3, 4].

In addition to a more favorable life cycle analyses, White Biotechnology processes like the BioPDO™ process demonstrate many advantages over petrochemical processes, such as the ability of the process to maintain high specificity [5], high yield, and ability to maintain lower concentrations of chemical intermediates that are generally recognized to form undesirable impurities in the final product [6].

Although many advantages exist for bioprocesses, disadvantages include (a) low to moderate productivity rates requiring intensification capital investments related to equipment capacity, (b) process susceptibility to bacterial and bacteriophage contamination, which has a negative impact on operability factor, (c) generally higher variable cost of manufacturing due to high electrical and related energy costs, (d) high water use, (e) odors, (f) costs associated with the production and filtration of large volumes of gases required for the fermentation process, and treatment of waste gases to remove odors, viable cells, and regulated or unregulated chemicals, (g) higher separation and refining costs due to the presence of large amounts of water, and (h) disposal of large volumes of nonhazardous waste.

In this chapter, we provide a detailed analysis of one of the most challenging issues for white biotechnology processes, which is the prevention of lytic bacteriophage events in commercial fermentors. Bacteriophages can cause rapid lytic infections of the highly clonal bacterial populations that are used in white biotechnology processes. Lytic infections of bacteria by bacteriophage reduce or abolish product productivity, and reduce the efficiency of cell recovery methods, which causes reduced product quality or a complete loss of the product. Batches affected by lytic bacteriophage infections cause a loss in production capacity or asset utilization, and financial losses to a business [7]. Lytic events in industrial fermentation may necessitate temporary shutdown of the facility for cleaning and elimination of bacteriophage, or even prolonged shutdown periods for cleaning and modification of aseptic barriers in the facility. In addition to surveillance and elimination best practices, this chapter outlines facility design considerations that are important in the prevention of bacteriophage in a biomanufacturing facility.

2. Prevalence of bacteriophage contamination in industrial fermentation processes

With the steady increase in the use of prokaryotic biocatalysts over the course of the last several decades for protein, small molecule, and chemical production, a focus has been placed on maintaining a bacteriophage-free environment in the manufacturing facility. The prevalence of bacteriophage in commercial fermentation processes varies considerably within the industry. For example, there have been no reported bacteriophage-related losses in certain industrial process, including: (a) syngas fermentations utilizing *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, and *Clostridium coskatii*; (b) the commercial process for production of Spinosad and Spinetoram using *Saccharopolyspora spinosa*; and the commercial process for (c) production of xanthan gum by *Xanthomonas campestris*. In contrast, moderate to severe fermentative losses have been observed for certain

bacterial-mediated processes, including the acetone-butanol-ethanol process that uses *Clostridium acetobutylicum*, *Clostridium beijerinckii*, or *Clostridium* sp. [8]; the *Escherichia coli*-mediated NutraSweet® process [9]; the *E. coli*-mediated BioPDO™ process [10]; the vinegar fermentation processes that uses *Acetobacter europaeus*, or *Acetobacter* sp. [11]; and food processes that utilize various bacteria [12, 13].

Although no single factor appears to link processes that are susceptible to bacteriophage-induced fermentative losses, some common themes exist:

- a. Tanji et al. [14] proposed that “the most likely source of phage contamination in the *E. coli* culturing process is human, since *E. coli* is one of the main inhabitants of the gastrointestinal tracts of warm-blooded animals.” Infection of microflora present in fermentation plant personnel may serve as a reservoir to maintain bacteriophage in the plant environment for extended periods of time [15]. Production hosts that are incapable of human colonization or that exhibit distinct phylogenetic differences to the natural human microflora may be advantaged over *E. coli* production hosts for these processes.
- b. Certain processes using *E. coli* have a distinct disadvantage that aseptic design and operation principles are unknowingly sacrificed by continuous improvement programs, which focus on minimizing process cycle time, or related cost reductions. Processes that produce end products that are inhibitory to foreign bacterial growth are especially vulnerable since a false sense of security is provided by the fact that the *E. coli* production hosts exhibit improved competitiveness over most other microorganisms. Under these conditions, foreign growth, which may harbor bacteriophage, can go undetected or unreported. Process cycle time improvements typically target a reduction in maintenance tasks supporting sterile barriers, or a reduction in time allotted for sterilization and clean-in-place (CIP) procedures to clean piping, valves, and vessels. These efforts can negatively impact bacteriophage elimination programs.
- c. *Xanthomonas campestris* has a number of established bacteriophages that have been registered as biocontrol agents to minimize its impact as a plant pathogen [16]. Therefore, the absence of bacteriophage-induced losses for the commercial xanthan gum process is aligned with the absence of an appropriate reservoir in the manufacturing facility that ecologically supports *X. campestris* growth.
- d. Microorganisms with doubling times greater than 2–3 hours, which includes *Saccharopolyspora spinosa*, *Clostridium ljungdahlii*, and *Clostridium autoethanogenum*, often exhibit effective DNA restriction-modification systems that serve to destroy foreign DNA that enters the cell. These microorganisms are typically referred to as recalcitrant to recombinant DNA technology, as measured by low or unmeasurable transformation efficiency [17]. More recently, a number of widespread bacteriophage immunity systems have been described, some of which contain elements that act similarly to restriction-modification systems, to modify or destroy both foreign (i.e., non-native plasmid or cosmid-associated DNA) and bacteriophage-associated DNA [18, 19]. Interestingly, the *E. coli* production host used for the BioPDO™ processes was found to lack a functional bacteriophage exclusion operon (BREX [18]), as well as a functional CRISPR-Cas restriction-modification system [7, 20]. Highly optimized strains utilized in commercial manufacturing process often contain intended and unintended modifications to the host chromosome that contribute to improved rate,

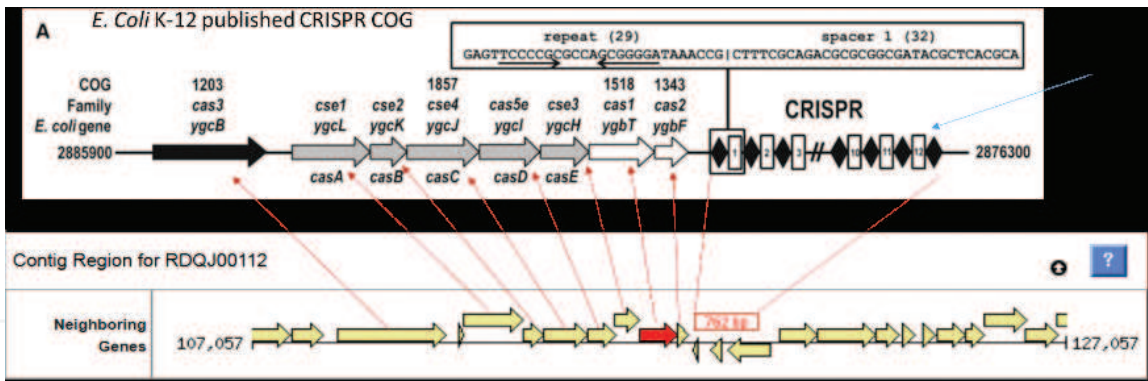


Figure 1. Alignment of the *E. coli* K12 CRISPR operon (upper panel) with the native nonfunctional CRISPR-Cas operon in the BioPDO™ *E. coli* production host (lower panel).

titer, and yield. Although the BioPDO™ production host contains nearly a complete *E. coli* K12 CRISPR-Cas operon (**Figure 1**), upstream regulatory modifications, which were correlated with improved product production rate resulted in the loss of function for this operon. Vale et al. [19] found that “Cas protein expression is particularly costly, as Cas-deficient mutants achieved higher competitive abilities than the wild-type strain with functional Cas proteins.” Smaller spacer libraries of approximately four spacers or less were not associated with fitness costs, suggesting that the genetically engineered spacer library approach of Halter and Zahn may serve to minimize energy and ATP burden on the cell, as indicated by the minimal impact of recombinant seven-spacer system on 1,3-propanediol biosynthesis [7].

2.1 Isolation and identification of bacteriophage DNA from lytic production samples in the BioPDO™ process

Lytic bacteriophage infection with the BioPDO™ process is characterized by sudden cellular lysis, which coincides with a sudden and rapid increase in dissolved oxygen (to 100% dissolved oxygen), a complete loss of oxygen uptake rate, and a complete loss of carbon dioxide evolution rate during the fed-batch fermentation. Optical density (OD) of the fermentation decreases rapidly from an OD₅₅₀ nm at approximately 11 hours of 42 ± 2 absorbance units to less than 1.4 ± 0.5 in a period of 30–40 minutes. Viable cell counts for the process show that a nearly complete 10-log reduction occurs within this time period. As in-house molecular techniques for bacteriophage detection were not initially developed at the time of facility start-up, these early events were poorly characterized. However, procedures existed to sample and preserve fermentor samples in segregated freezers to support future investigative efforts. Fermentor samples that were collected consisted of a crude mixture of cleared (lysed) *E. coli* cells, nucleic acids, proteins, and cell membrane components. These samples were subsequently determined to contain bacteriophage particles, which were directly observed by transmission electron microscopy (**Figure 2**).

2.1.1 DNA isolation methodology

Samples were syringe-filtered through a 0.2-µm filter to separate the larger cell debris, and bacteriophage particles were precipitated and concentrated for transmission electron microscopy (**Figure 2**). To improve DNA sequencing efforts,

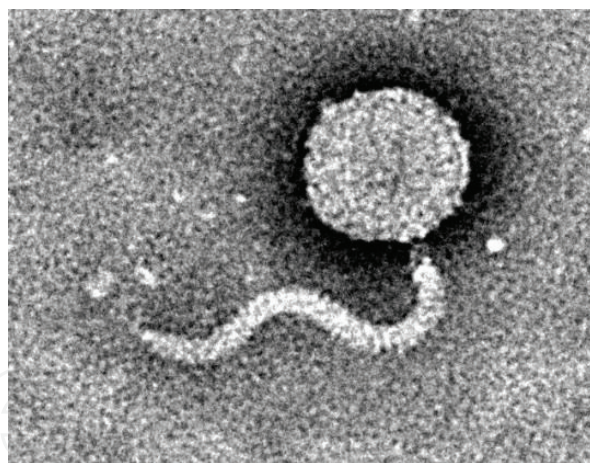


Figure 2.
Transmission electron micrograph of bacteriophage DTL.

bacteriophage nucleic acids were separated from lysed *E. coli* DNA. The bacteriophage DNA is protected by the proteinaceous capsid head, allowing for DNase digest of exogenous *E. coli* DNA. Filtered samples were incubated with DNase I according to the manufacturers recommended protocol overnight, ensuring complete digestion of all DNA present that could interfere with bacteriophage DNA sequencing. After complete digestion, the DNase was deactivated by heat denaturation. The next step was the removal of the bacteriophage capsid protecting the DNA. This was performed by treatment with proteinase K according to the manufacturer's protocol for 3 hours. The proteinase K treatment removes the capsid protein shell, allowing the bacteriophage DNA to enter solution. Now that the bacterial DNA was fully degraded and the bacteriophage DNA removed from the particle capsid, the final remaining steps were simply precipitating the remaining protein from solution, separation from the aqueous phase by centrifugation, and precipitation of nucleic acids. Protein precipitation was performed by addition of 2 M potassium hydroxide. Immediate flocculent formation was evident, but the samples were stored on ice for a short period to encourage further protein precipitation. Chilled samples were centrifuged in a tabletop centrifuge at $13,000 \times g$ for 10 minutes to separate the protein flocculent from the nucleic acid-containing supernatant. Upon separation, nucleic acids were precipitated through a 50% isopropyl alcohol wash. Samples were again stored on ice to allow precipitation to progress more efficiently, followed by centrifugation at $13,000 \times g$ for 5 minutes. The visible, cloudy DNA pellet was resuspended in ddH₂O and quantified for sequencing.

2.1.2 Bacteriophage typing

The isolated DNA was sent for 454 pyrosequencing, which revealed an approximately 46,000 base pair circular genome. The genomic characterization of the plasmid was described previously [7]. Sequence analysis of genomes isolated from lytic batches ranging back to the manufacturing facility start-up in 2006 revealed an interesting fact that every lytic bacteriophage event was caused by the same bacteriophage. This bacteriophage shared sequence homology with a T1-like bacteriophage, referred to as RTP bacteriophage [21], but sequence homology of tail fibers and other open reading frames in the genome indicated it was a new representative of this group [7]. To reflect the source of the novel bacteriophage, it was named "DTL-phage," for the location of its discovery at the DuPont Tate & Lyle Bio Products fermentation manufacturing site. It is noteworthy that with the significant diversity of coliphages in nature, only one specific type of bacteriophage was

detected over a period of 12 years. The prevalence of this particular bacteriophage at this facility was likely enhanced by the lack of significant changes in bacteriophage resistance in the *E. coli* host strain, and the massive amount of bacteriophage particles that was released from the site's five—600,000-L production fermentors. With a burst size of approximately 300 new phage particles with each cell lysis, infected fermentors were estimated to release approximately 2×10^{15} viral particles per affected fermentor, which were then distributed to vent and broth deactivation systems where these phage particles were presumed to be deactivated. Subsequent viral load studies showed that large amount (40–65%) of the bacteriophage present in liquid and respiratory off-gas analysis streams remained active. Although a majority of flow in these systems was contained, broth from in-process fermentor sampling efforts, and a majority of excess flow from off-gas analysis streams for respiratory gas analysis was not contained, and contributed to bacteriophage-containing aerosols, and dried particulate matter, which was disseminated in the production facility.

While we have shown that bacteriophage *DTL* is highly selective for *E. coli* (**Figure 3**), it is capable of lysing several different *E. coli* Group A representatives and related subspecies (**Figure 4**), suggesting that bacteriophage particles could infect related *E. coli* subspecies present in the local manufacturing environment, since these representatives are common human and animal inhabitants [14].

Since the bacteriophage plaque assays provide slow (34–48 hours) feedback for the presence of bacteriophage contamination in process samples, development of a rapid diagnostic test was considered a critical path for effectively mitigating fermentative losses in the facility. To this end, polymerase chain reaction (PCR) assays were developed to detect *DTL*-phage nucleic acids in fermentation broth (**Table 1**). Primer sets were designed based on the genome sequence ([22]; Genbank accession number MG050172), and endpoint reactions performed on both lysed and nonlysed fermentation samples were run on 1% agarose gel, providing a binary visual confirmation of the presence or absence of *DTL*-phage genomic DNA (**Figure 5**).

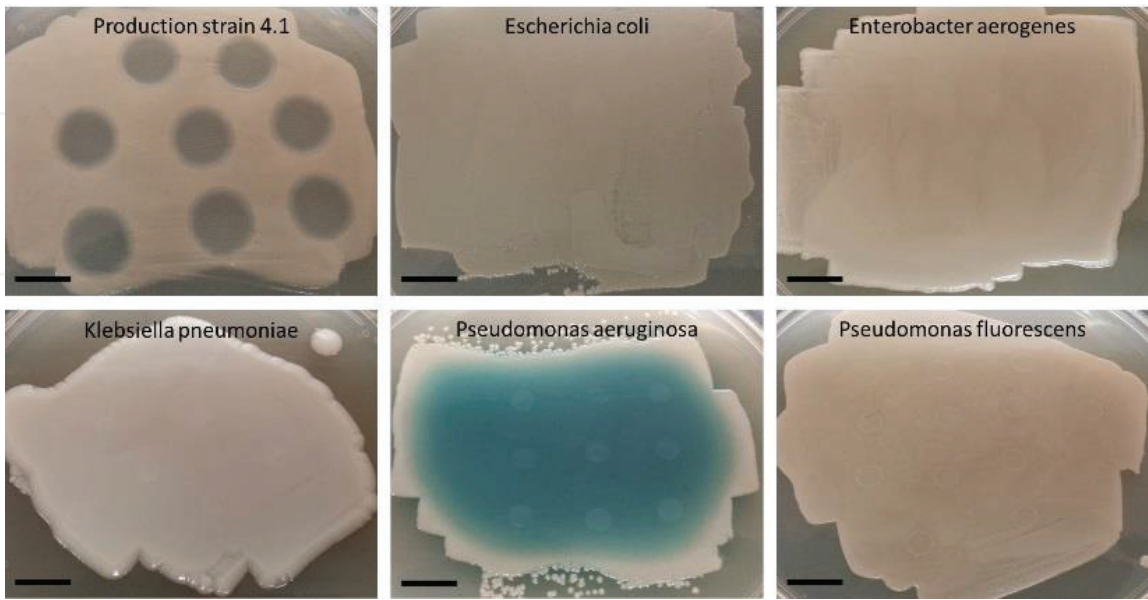


Figure 3. Plaque assays using *DTL*-phage on phylogenetically diverse bacteria. From the top panel—left to right: *Escherichia coli* FM5, *E. coli* ATCC 8739, *Enterobacter aerogenes* ATCC 13048; from the lower panel—left to right, *Klebsiella pneumoniae* ATCC 15574, *Pseudomonas aeruginosa* ATCC 9027, and *Pseudomonas fluorescens* ATCC 13525. Background spotting, which is most notable with *P. aeruginosa* is due to nutrient carry over in the phage preparation.

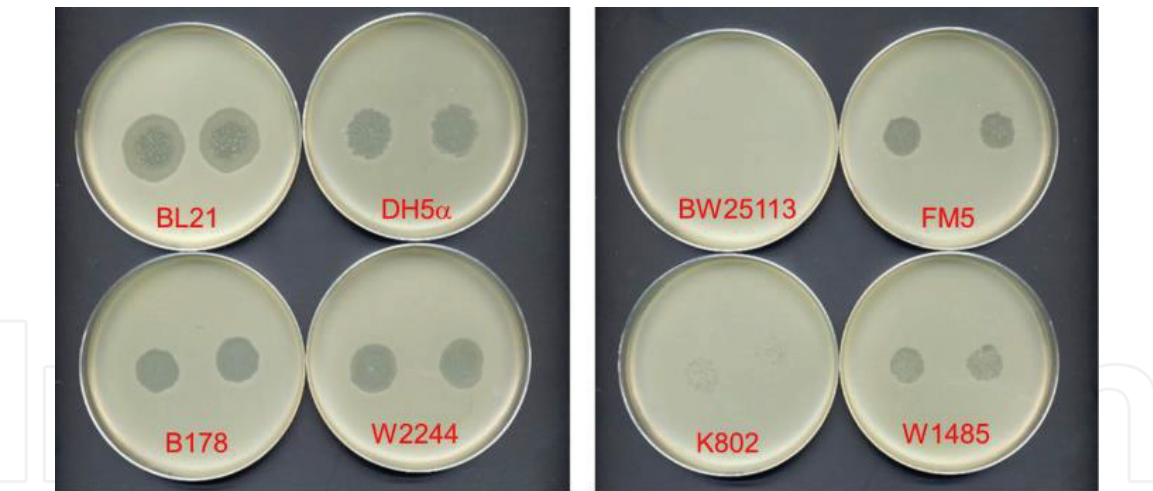


Figure 4.
Susceptibility of Escherichia coli group A representatives and related E. coli subspecies to DTL-phage. Susceptible = BL21, DH5α, B178, W2244, FM5, and W1485. Nonsusceptible = BW25113 and K802.

Primer	Sequence
DTL-phage 43 qRT F	GAAGAGGTGTTTAATTTTCGCCG
DTL-phage 43 qRT R	GCCAAACCCGTTAATGTGAAC
DTL-phage 40 qRT F	AGAGGTAGTGGTAGGTTCCG
DTL-phage 40 qRT R	TCAAGAATCGCAGAGTAACCG
DTL-phage 19 qRT F	GCACGCTGGTTAATGGAATG
DTL-phage 19 qRT R	TTCTTGATGGAGATTGTCGGG
DTL-phage 6 qRT F	GGCGTAAAACAGTAATTCAGGTC
DTL-phage 6 qRT R	TTCACACCATCACTACCATCAG
DTL-phage 66 qRT F	GCAGTAAGCCAGAGATTAGCG
DTL-phage 66 qRT R	CTATCCAGTGACCCAACCTTG

Table 1.
DTL-phage primer sets for PCR diagnostic testing.

After optimization of the assay, early PCR screening of every fermenter was implemented in the manufacturing facility for transfer of seed fermentors. It was soon clear that endpoint PCR, followed by gel-electrophoresis was still too slow to effectively meet the 2-hour transfer window in the manufacturing process, and secondly, the technical tasks and decisions were too complex to effectively implement for the nontechnical manufacturing facility workforce. Real-time quantitative PCR is an assay that provides a more rapid result (<1 hour), and also produces a threshold cycle (C_t) count that can be used as a straightforward quality control parameter to set a simple quantitative threshold for amplicon copy numbers. The use of real-time quantitative PCR to track fermenter contamination provides a more rapid screening procedure and reduces the time from fermentor sampling to a formation of a transfer decision by slightly over 1 hour. Primer sets were optimized to amplify targets within the bacteriophage *DTL* genome with above 95% efficiency, and premade reaction cocktails were also developed to minimize low-volume pipetting steps needed to be performed by nontechnical staff. This procedure had the following steps: (a) A seed fermenter sample would be collected by the fermentation technician and transferred into

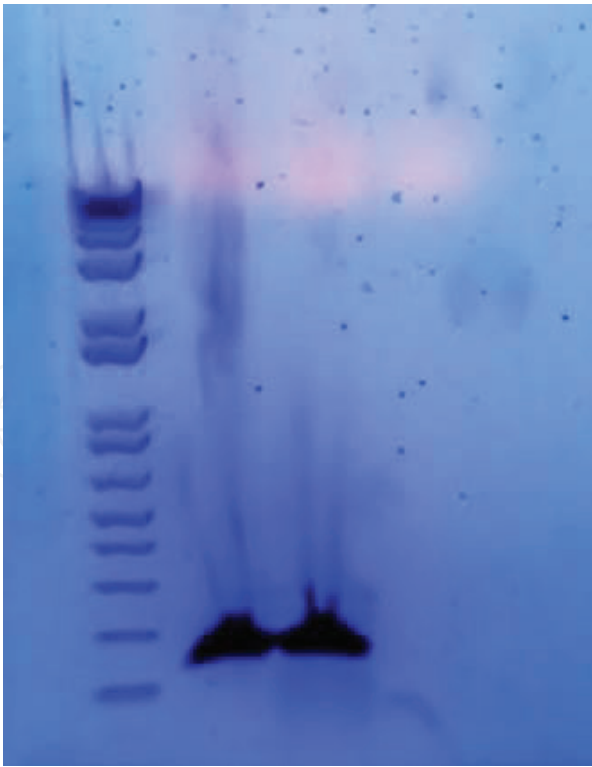


Figure 5. DTL-phage amplicons generated from PCR (DTL-phage 40 set, **Table 1**) and separated by 1% agarose gel electrophoresis. Lane 2 is a positive control of purified DTL-phage DNA, lane 3 is a seed fermenter sample containing 2×10^4 particles of DTL-phage per mL, and lane 4 is a sterile water negative control. Lane 1: Invitrogen E-Gel 1 Kb Plus Express DNA Ladder (part #10488091) 100–5000 bp.

a class 2 biological safety cabinet. (b) The sample would be filtered through a 0.2- μ m sterile syringe filter to remove intact cells, and then diluted 1:10 in sterile water. This dilution was performed to dilute inhibitory substances present in the fermentation media, allowing for elimination of the DNA extraction step. (c) Load the processed sample with positive and negative controls into the quantitative PCR instrument, and initiate the analysis. This qPCR assay was the cornerstone in a strategy to screen seed fermentors prior to transfer to the production fermentor as a safeguard to limit bacteriophage-infected seeds from contaminating production fermentors. This new procedure included control charting intensities for C_t trends, which aided in the tracking of bacteriophage contamination levels (**Figure 6**), which increased the operability factor for the

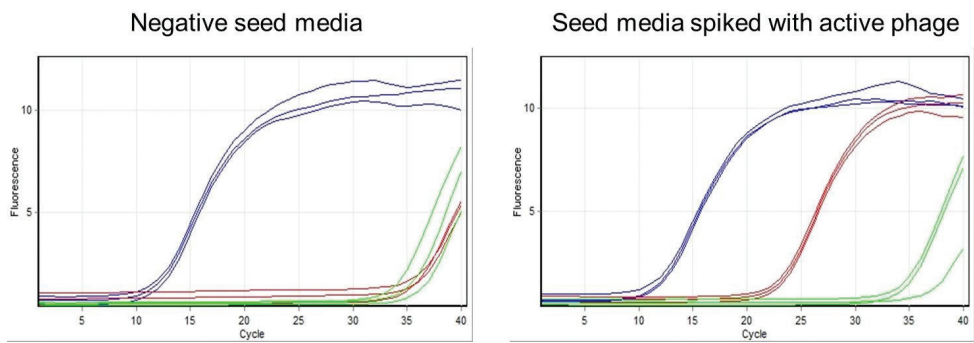


Figure 6. Quantitative PCR (qPCR) amplification curves of seed media screened for DTL-phage. Blue colored curves represent a positive control for presence of DTL-phage (equivalent to 180 plaque forming units per milliliter), green curves represent a negative control, and red curves represent the seed media being screened. The left panel shows a negative result for the seed fermentation sample with no detectable DTL-phage, and the right panel shows a positive response for a seed fermentation sample that contained an internal addition of 80 plaque forming units per milliliter.

fermentation process by ensuring that only bacteriophage-free seed material was transferred into production fermentors.

2.2 Efforts to produce a bacteriophage-resistant production strain

There is a clear incentive for businesses that utilize fermentation-based manufacturing technologies to invest in the development of production strains that are resistant to potential bacteriophage threats. There are at least four target areas that have been described in literature for enhancing bacteriophage resistance in prokaryotic organisms that are sensitive to lytic bacteriophage infections [23]: (a) prevention of phage adsorption, (b) preventing phage entry, (c) cutting bacteriophage nucleic acids, and (d) abortive infection systems. The following analysis will summarize efforts in two of the four areas and prevention of phage adsorption and cutting phage nucleic acids with a heterologous CRISPR-Cas system.

Classical strain improvement programs for the generation of bacteriophage resistance often start with acquiring genotypic diversity in a host population through spontaneous or induced mutation, followed by challenge and selection of survivors to bacteriophage infection. Several studies utilizing this strategy have reported resistance through single nucleotide polymorphisms (SNPs) or insertional mutagenesis, which often alters the structure of bacteriophage binding sites on the cell surface. Additionally, significant deletions of chromosomal DNA have been described that involve structural changes of one or more cell surface biomolecules [14, 24–26].

The initial step in the bacteriophage virulence cycle is the adsorption of the bacteriophage particle to the outer surface of the bacterial cell [23]. Once bound, the bacteriophage infects the cell by transferring genetic material into the cell, where it then utilizes host cellular translation systems to generate additional bacteriophage particles. This binding of the bacteriophage to the outer membrane is typically mediated by a highly specific receptor on the outside of the cell, is typically a membrane protein, a specific class of lipid, or a carbohydrate moiety, and is extremely specific to a bacteriophage and its host. Common genetic techniques can, therefore, be used to target these binding sites to reduce binding affinity or eliminate the site [24, 26].

Over several years, classical strain improvement programs for the BioPDO™ process have generated and screened strains that were selected through challenge for resistance to DTL-phage [7]. These screens included strains that demonstrated a 3–5-log improvement in the reduced sensitivity as measured by the standard bacteriophage plaque assay (**Figure 7**). In all cases, bacteriophage resistance was associated with a 63–96% reduction in 1,3-propanediol production titer. Further analysis showed that these strains had a lower solvent tolerance, which indicated that the bacteriophage resistance mechanism(s) were presumably linked to changes in cellular ultrastructure. Genomic sequencing efforts confirmed that SNPs occurred in a series of genes involved in synthesis and glycosylation of lipopolysaccharide, including glycosylation with heptose residues [27]. Heptose residues appear to be essential for high-affinity binding of DTL-phage to the *E. coli* cell [28].

The second strategy, cutting bacteriophage nucleic acids is a rapidly evolving field that has been heavily influenced by recent discoveries in bacterial-acquired immunity [12]. Clustered regularly interspaced short palindromic repeats (CRISPR) are a molecular system by which prokaryotes obtain acquired resistance to bacteriophages. The CRISPR operon is widely distributed in prokaryotes and represents the most abundant form of innate immunity in these organisms [29]. Upon injection of the genetic material, the cell recognizes foreign DNA, and the first gene involved



Figure 7.

Bacteriophage plaque assay plate showing the presence of *E. coli* colonies growing in the zone of clearing that are resistant to DTL-phage challenge.

in the CRISPR pathway creates an approximately 20–25 base pair cut (this varies between species and CRISPR subtypes). This snippet of DNA is then integrated into a library of recognized bacteriophage genetic elements, flanked on each side by a palindromic repeat. This genetic element can then be transcribed, producing a single-stranded transcript that is homologous to the DNA of the intruding bacteriophage. The palindromic repeats flanking the element serve to bind the short fragment of transcript to a nuclease, which is then guided by the homologous element to potential binding sites on the intruding segment of DNA, where a single cut is made rendering it nonfunctional.

Since it was established earlier that the BioPDO™ *E. coli* production host did not carry a functional CRISPR operon in its genome, and the specific binding site of the DTL-phage was originally unknown, work was initiated with CRISPR to generate a spacer library specific to DTL-phage infection. The well-characterized CRISPR cassette of *Streptococcus thermophilus* was amplified, and its 12 bacteriophage spacers (remnants of previously acquired resistance in this species) were replaced with seven spacers that were homologous to different regions of the DTL-phage genome. These seven spacers were specifically chosen to deliver CRISPR-nuclease-delivered cuts in the middle of important open reading frames within the bacteriophage genome, thereby ensuring overwhelming and targeted knockout of its genetic functionality.

Upon incorporation of this tailored cassette to the BioPDO™ *E. coli* production strain, plaque assays and phage challenges indicated the organism had obtained full resistance to this bacteriophage, as indicated by the lack of plaques formed in experiments using phage titers as high as 10^6 phage particles per milliliter. The utilization of multiple homologous spacers was an important aspect of this project to reduce the potential for resistance. Because CRISPR resistance depends on homologous binding of spacers bound to the CRISPR nuclease to the intruding phage DNA, a single SNP acquired within the homologous stretch of DNA in the phage genome could disrupt the DNA-nuclease interaction, rendering that spacer no longer active. The use of seven targeted spacers minimizes the potential for resistance, and no significant energy or ATP burden related to the lack of fitness hypothesis, proposed by Vale et al., was observed [19]. Although Vale et al., proposed a threshold number of four spacers as the limit regarding negative impacts on cell fitness, the slight increase to a seven-spacer library appeared to have no negative consequences on 1,3-propanediol synthesis rate [7].

2.3 Reservoirs and process entry points for bacteriophage in a manufacturing setting

Host resistance to bacteriophage infection is only a partial solution to the prevention of bacteriophage in fermentation processes. This approach must be balanced with effective facility sanitation procedures, active bacteriophage surveillance programs that are coupled with reservoir elimination efforts, and finally, the validation, maintenance, and monitoring of aseptic barriers utilized in the process for bacteriophage exclusion.

As mentioned previously, bacteriophage-infected production fermentors for the BioPDO™ process were estimated to release approximately 2×10^{15} viral particles per affected fermentor based on a 300-burst size model. Bacteriophage-contaminated air and liquid in fermentors was transferred to vent and broth deactivation systems where the viral particles were presumed to be deactivated. Earlier pilot plant studies of microbial and biological materials deactivated by a high-temperature short-time sterilizer (HTST) did not consider bacteriophage inactivation because (a) bacteriophage was considered an addressable nuisance in the operation of the facility, (b) bacteriophage libraries specific to the BioPDO™ production microorganism had not been assembled, and (c) risks to a commercial process remained largely speculative. Furthermore, the closest-related bacteriophage, RTP-phage had not been isolated and described until after the plant start-up in 2006. Subsequent heat-inactivation studies on phage-contaminated batches after facility start-up were successful in showing that bacteriophage particles were not completely deactivated in the HTST broth deactivation system, mainly due to resistance to heat inactivation [30, 31].

Other weaknesses in containment systems were gas and liquid sampling systems, which were commonly used in the fermentation industry for respiratory gas analysis and control through feedback loops, and in-process analytical for measuring broth parameters associated with growth and product production, respectively. Once free of containment systems, the bacteriophage particles were unintentionally disseminated by human activity and other environmental mechanisms. This disseminated material served to challenge sterile barriers that were in place for air filtration, filters used for sterile filtration of liquids used in the process, and cross contamination of microbiology process laboratories, which impacted the seed train, and laboratory diagnostic tests.

The ecological aspects of DTL bacteriophage are poorly characterized due to the lack of historical environmental samples from the fermentation facility during the period of the initial infection. We are in agreement with the proposal of Tanji et al. [14] that the most likely source of phage contamination for an *E. coli* culturing process is human, and the initial source of DTL-phage in the BioPDO™ facility, was most likely also human. The fact that DTL phage is now prevalent in the plant environment is thought to be mainly a result of a series of lytic events in production fermentors that established localized concentrations of the bacteriophage in reservoirs that are inhabited by natural populations of *E. coli*, or closely related microorganisms that are susceptible to this bacteriophage.

One of the most significant failure points in the commercial fermentation facility process for bacteriophage entry was through sterile filtration barriers. The small size of DTL-phage (capsid diameter = 0.075 µm) supports passage of the virus through liquid-service filtration systems (0.2–0.45 µm cutoff size), and conditional passage through gas-phase filtration systems (0.2 µm cutoff size) when entrained liquid is present. Aerial transfer of bacteriophage particles is well-established as a route for contamination of industrial fermentation processes [29, 30]; therefore,

air filtration systems play a large role in protecting the fermentation process from airborne bacteriophage particles.

Our empirical studies on the Donaldson sterile air filter, model P-SRF N 30/30 (0.2 μm absolute, expanded PTFE filter membrane) indicated that the filter was 100% effective in rejection of DTL-phage particles if the filter remained above the system pressure dew point temperature of 102.5°F (**Figure 8**). Since the design temperature of air present in the header was 122, the 102.5°F pressure dew point was considered to have sufficient safety factor to prevent formation of condensate. Wetting of the filter was correlated strongly with lytic infections by DTL-phage [7]. Wetting of the sterile filter due to entrained water in the process air header was infrequently observed when: (a) low ambient temperatures cooled areas of the process air header that were not adequately protected with heat tape and/or insulation (**Figure 9**), (b) cooling tower water or chilled water from the intercooler and/or aftercooler heat exchangers on the air compressor leaked into the air stream and traveled through the air header to prefilters and sterile filters, (c) low-point zones in the process header not adequately drained with autopurging condensate traps, and (d) high-temperature excursions of the chilled water system, which was used to condense water in the compressed air product stream. A critical factor for surveillance and prevention of bacteriophage contamination was the integration of dew point sensors at upstream locations from the sterile filter, and more frequent sterilizations of the filters in the sterile filter housing. Since sterile filters have a limitation of approximately 170 steam cycles, more frequent sterilization served to increase the number of filter replacements and overall process costs.

Bubble column bioreactors used in aerobic fermentation processes require large volumes of air to meet the oxygen uptake requirements for *E. coli* cells in the fermentation, as well as a means for mixing to drive heat transfer, gas solubility, CO₂ ventilation or removal, and mixing of substrates, and other feeds. Due to the expense of water vapor removal in very large volumes of air, many fermentation systems utilize ANSI/ISA-7.0.01-1996 air quality class 6 or lower specifications, which establish a dew point of 50°F at atmosphere pressure. This type of air compressor system, which contains chilled fluid after-coolers, passes the compressed air through a cooled heat exchanger, causing water vapor to condense out of the air stream, and typically produces air with a dew point not lower than 41°F (5°C). The pressure dew point refers to the dew point temperature of a gas under pressure, which in this case is increased to a value of 102.5°F at a pressure of approximately 84.7 psia (**Figure 9**). The main issues with this system are (a) the potential for



Figure 8.

Sterile process of destructive disassembly a Donaldson sterile air filter, model P-SRF N 30/30 (0.2 μm absolute, expanded PTFE filter membrane) for bacteriophage diagnostic testing. Note the dark water staining on protective prefilter barrier layer on the filter.

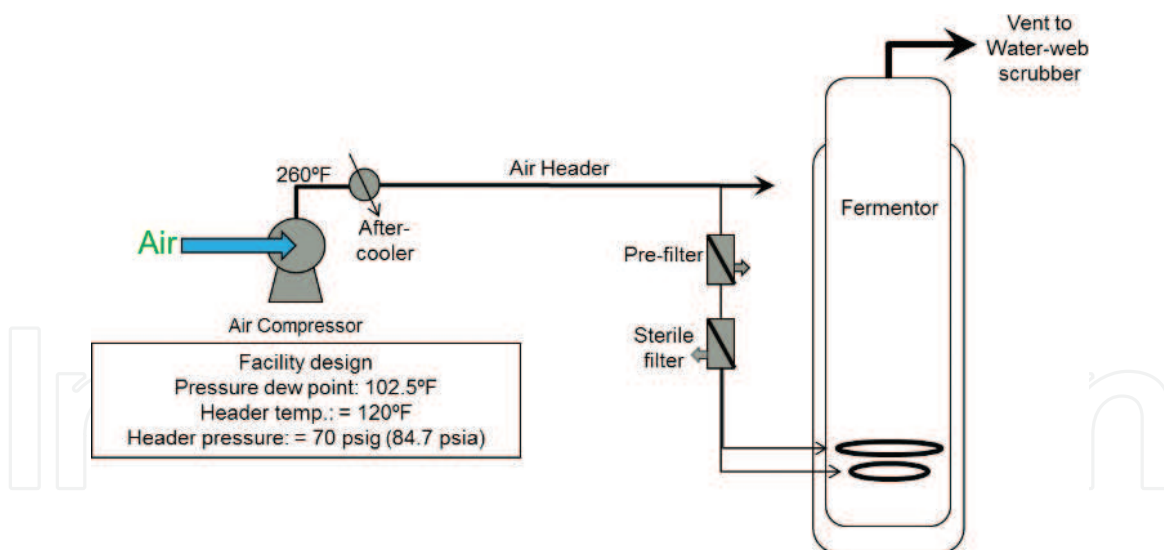


Figure 9.
 Diagram of the process air system that provided a sterile-filtered air to a production fermentor.

heat exchanger leakage into the air stream and (b) temperature excursions of the cooling fluid (chilled water), due to its use in other areas of the manufacturing facility. Temperature excursions for chilled water were found to reduce the ability of the after-cooler to remove water vapor in air supplied to the fermentors, which increased the potential for condensate to form in the process air header. This condensate was found to cause passage of bacteriophage particles through the sterile, 0.2- μ m air filter.

Deviations from aseptic design principles are nearly always a root cause for introduction of foreign bacterial growth and bacteriophage into axenic fermentation systems. A deviation in these principles occurred as part of the risk mitigation effort to prevent condensate formation in the process air header. As part of this project, sterile air bypass lines were installed on the air header line to eliminate zero air flow conditions that occurred during the sterilization cycle for fermentors. During fermentor sterilization, sterile air flow was stopped, and a 45 psig steam feed line between the sterile filter housing and fermentor was opened. During the 90-minute sterilization cycle, the temperature of the air header was found to drop well below the pressure dew point, especially during cold weather months, which introduced condensate upstream of the sterile air filters. Introduction of a process steam line upstream of the sterile filter housing, as is done more commonly in the industry, was not considered a best practice because the sterile air filter would remain wetted for a short period after the steam sterilization process was completed, and nonsterile air flow was reinitiated as a feed stream to the filter housing. The wetted filter was believed to promote passage of bacteriophage particles into the fermentor. One solution, practiced at a separate facility, was to have a secondary low-flow sterile air header upstream of the main sterile filter housing that provided sterile dry air to the housing after the steam sterilization to dry the wet sterile filters. As these former solutions required significant down time to perform repiping, two other solutions were prioritized: (1) ensure that heat tracing and insulation of the air header was sufficient to maintain temperature above the pressure dew point and (2) prevent the low or no-air-flow condition by adding a small diameter purge line directly after the sterile air filter housing to provide constant flow in the air header. Due to the potential for ammonia gas in the bypass air stream, piping effort focused on connection of this line to the fermentor exhaust header, which contained an ammonia scrubber system. The new piping system did not contain proper aseptic barriers and steam-purged interlocks, which then permitted backflow of

contaminated waste gas into the fermentor air header (poststerile filter) under infrequent vent header over-pressure events.

One of the more surprising discoveries in this work was the level of bacteriophage contamination (up to 7×10^3 plaque forming units per square centimeter) in the process air header, as well as the surface of sterile air filters between sterilization cycles. This result indicated that the extremely short, but elevated air temperature of 260°F between the air compressor and after-cooler was insufficient in deactivating DTL-phage (**Figure 9**). Based on this finding, air header sanitation procedures were developed to infrequently steam sterilize the air header for the location between the preair filter housing and the air compressors. While this practice reduced phage burden in the air header, it could not be operated in a continuous mode, and therefore, the sterile air filters served as the only barrier to bacteriophage entry. Additional design improvements related to a secondary barrier for airborne phage entry include: (a) a heat-sterilization system on the product side of the air compressor to elevate the temperature to 300°F, and increase temperature hold time by 10-fold to 1 second, and (b) filtration/precipitation systems for pretreatment of supply air to the air compressors.

Generation of bacteriophage aerosols in the BioPDO™ manufacturing facility is largely driven by failure points in containment systems, including the industry-standard water-web hypochlorous acid scrubber system for fermentor vent stream treatment, as well as the use of open liquid transfer systems, such as trench drains that are used for streams originating from steam interlocks on sample systems, drains from sample sinks, drains from vapor-liquid separators, and unintended leaks from heat exchangers and piping. Efforts to ensure that these streams are transferred by closed piping systems to chemical or steam deactivation systems is critical for achieving low airborne concentrations of bacteriophage in the manufacturing environment. Frequent cleaning of these areas with disinfectants has been shown to be critical for limiting dispersion in the manufacturing facility environment [32].

3. Conclusions

As the most abundant biological entity on the planet, bacteriophages play an important ecological role in ecosystems and have also been exploited for the development of many modern technologies, including gene transfer and treatment of bacterial infections [7, 14, 21, 23]. The presence of bacteriophage in an industrial fermentation facility can be a serious problem, resulting in reduced product quality, loss in production facility operability, and financial losses to a business. In this chapter, we have described basic and applied research efforts around our goal of reducing the impact of bacteriophage-related losses in a commercial process for the manufacture of 1,3-propanediol. The key to success in these efforts was the development of rapid diagnostic methods that were subsequently leveraged by a diverse team to quickly diagnose and eliminate sources of bacteriophage in a fast-paced manufacturing environment.

Many of the solutions related to operating a manufacturing facility bacteriophage-free require participation of a cross-disciplinary team that encompass many areas of expertise, including virology, microbiology, microbial ecology, chemical, and mechanical engineering. As with any new biomanufacturing process, designers utilize a basic set of assumptions that serve in the design and construction of the facility. Some of these assumptions are not fully tested in a pilot plant, and the issues that arise are often seen for the first time in the commercial fermentation facility. In this chapter, we have characterized issues related to the use of 0.2 µm (absolute)

sterile filters as the only mechanism for exclusion of bacteriophage from the fermentation process. This issue is especially problematic since these filters were not specifically designed for removal of irregular bacteriophage particles with diameters (0.075 μm), which is significantly smaller than the stated filter cutoff size.

Our studies indicate that CRISPR/Cas9-mediated resistance to bacteriophage DTL in the *E. coli* PDO production strain is a highly effective strategy for eliminating bacteriophage virulence. The CRISPR spacer customization strategy further ensures that spacers are not generated against foreign DNA that is inserted into the production host as part of future host improvement (transformation) projects. Furthermore, the metabolic burden hypothesis proposed by Vale et al. [19] is avoided since we have limited the spacer library to a total of seven spacers. The main disadvantage of this approach is that this recombinant CRISPR system has a narrow range of bacteriophage specificity, and that there is a significant selection pressure for sequence modifications that are not recognized by this existing spacer library. In this regard, there is a difficult balance between spacer library size as an assurance of bacteriophage resistance, and acceptance of lower 1,3-propanediol productivity due to the metabolic burden due to the increase in spacer library size.

Conflict of interest

The authors declare that there are no conflict of interests with this work.

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